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Ascites 13762 cells, a highly metastatic and malignant rat mammary adenocarcinoma cell line, express a cell surface glycoprotein called ASGP2 that possesses an epidermal growth factor (EGF)-like domain believed to interact with and stimulate growth factor receptors of the ErbB family. These cells also express ErbB2, a member of the ErbB family whose aberrant activation probably plays a role in tumoigenesis and tumor progression in a significant subset of breast cancer patients. ASGP2 and ErbB2 exist in a complex at the surface of ascites 13762 cells, raising the possibility that these cells stimulate their own growth or malignancy through an autocrine mechanism involving the ASGP2/ErbB2 interaction. The overall goal of the studies outlined in this proposal was to test this hypothesis. Studies were aimed at examining the effect of ASGP2/ErbB2 complex expression on the growth and transformation of cultured tumor cells. We expressed ASGP2 in an ErbB2-expressing human melanoma cell line under an inducible promoter. We observed that the result of the interaction of the ASGP2 with the ErbB2 receptor is to potentiate the response of that receptor to EGF-like growth factors, both in receptor tyrosine phosphorylation and in the progression of the cells through the cell cycle.

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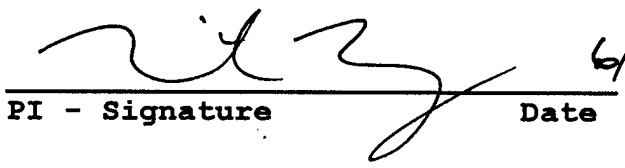

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Introduction

Epidermal growth factor (EGF) is the prototype for a family of ligands that influence not only the proliferation and differentiation of cells in normal tissues but also the growth and possibly the malignancy of cancer cells (1,2). Stimulation of normal cells and tissues by the EGF-like growth factors generally proceeds through endocrine or paracrine mechanisms, where the target cell is in a location distal to the cells secreting the diffusible growth factor. However, accumulating evidence indicates that autocrine, juxtacrine and intracrine mechanisms may also play roles in cellular growth regulation, particularly for tumor cells.

The EGF-like ligands exert their effects through the ErbB family of growth factor receptor/tyrosine kinases, which includes the EGF receptor, ErbB2, ErbB3 and ErbB4 (3). Overexpression of each of the members of the ErbB receptor family has been observed in human tumors, and it is suspected that the activated receptors participate in tumor formation and progression. Particular attention has focused on ErbB2 because the aberrant activation of this protein is known to be tumorigenic to transgenic animals. More importantly, the amplification of the *erbB2/neu* gene and the overexpression of the ErbB2 protein has been observed in a significant proportion of breast cancer patients. In fact, several studies suggest that the overexpression of $p185^{erbB2/neu}$ correlates with a poor patient prognosis for some subsets of patients (4-6). Since overexpression of the ErbB2 protein leads to its increased tyrosine kinase activity, these observations suggest that the aberrant activation of ErbB2 kinase activity directly contributes to breast cancer tumorigenesis and progression. Thus far, most studies concerning ErbB2 and breast cancer have been directed toward characterizing the amplification and overexpression of the *erbB2/neu* gene in tumors. The goal of such studies was to develop ragents that might prove useful in the diagnosis of malignant forms of breast cancer, based on the overexpression of the ErbB2 protein (4-6). It is clear, however, that overexpression of ErbB2 is only one of possibly many mechanisms for its activation. Indirect activation of ErbB2 by EGF and neuregulin (NRG) through heterodimerization with and cross-phosphorylation by other ErbB family members represents another mechanism for activation. These observations raise the question of whether or not a direct ligand for ErbB2 exists. Several activities have been reported but none have been cloned or characterized in detail. Most screens for activating ligands use either serum or conditioned media from cultured cells as starting material (7-9). However, given the diversity in mechanisms by which growth factors can act (juxtacrine, intracrine, etc.) it is possible that some activating ligands do not act as secreted factors, but are only active within the membrane environment. Because such proteins would possess hydrophobic transmembrane sequences, they may be difficult to identify, purify and characterize. However, such ligands could be potent activators of ErbB2 activity, and may be intimately involved in the contribution of ErbB2 to breast cancer.

Ascites 13762 cells are an autonomously proliferating, highly metastatic and malignant rat mammary adenocarcinoma cell line propagated in the rat peritoneal cavity. These cells express in abundance at their surfaces a complex of two glycoproteins, the ascites sialoglycoproteins ASGP1 and ASGP2 (10). ASGP1 is a sialomucin of molecular weight >600 kDa, over 400 kDa of which is O-linked oligosaccharide (11). Its abundance, large size and heavy glycosylation allows it to serve as an inhibitor of protein-protein and cell-cell interactions, as well as a screen from natural killer cells of the immune system (12-14). Although synthesized from the same message, ASGP2 has a very different character. It is a ~120 kDa glycoprotein consisting of predominantly N-linked glycosyl moieties, and is the membrane-bound component of the complex (15). Upon cloning and sequencing of the complex (16), it was found that ASGP2 possesses at least five identifiable subdomains: a very small intracellular domain of 20-25 amino acids, a single hydrophobic transmembrane domain, a cysteine-rich domain of unknown function, and two EGF-like domains that contain all the consensus amino acid residues characteristic of the active growth factors. Moreover, the spacing of cysteine residues in the EGF-like domain closer to the amino terminus of the protein (N-EGF) is very similar to those of the active growth factors.

These observations raised the possibility that the ASGP2 protein is capable of binding to and activating receptors of the ErbB family. Using insect cell technology, we have shown that ASGP2 selectively binds to ErbB2, that one of the EGF-like domains is responsible for this interaction, and that purified ASGP2 can stimulate the tyrosine kinase activity of ErbB2 *in vitro* (see Appendix). Our observations indicate that ASGP2 and ErbB2 are capable of forming a complex in cells that co-express the two proteins, and that this association can affect ErbB2 function. Considering that aberrant activation of ErbB2 is suspected to be involved in breast cancer progression, our observations point to the possibility that the expression of ASGP2 by breast cancer cells

might contribute to their autonomous growth or malignancy by stimulating ErbB2 activity. We propose that ASGP2 can act as an autocrine or intracrine regulator of ErbB2 in cells that co-express the two proteins, and that the expression of the ASGP2/ErbB2 complex stimulates cellular growth properties. The overall goal of the studies discussed below is to rigorously test this hypothesis by introducing the complex into cultured cells, and determining whether the cellular growth state is significantly influenced.

Results and Discussion

Specific Aim I: Analyze the capacity of ASGP2 to act as an activating ligand for the ErbB2 receptor when the two proteins are co-expressed in cultured mammalian cells.

On the basis of our previous observations, we propose that the co-expression of ASGP2 with ErbB2 enhances the tyrosine kinase activity and autophosphoryltion of that receptor. Our previous attempts to address this hypothesis in insect cells were unsuccessful because insect cell-expressed ErbB2 is constitutively active and fully autophosphorylated. To circumvent this problem, we employed a COS cell transient transfection system to assess ASGP2 effect on ErbB2 kinase activity. As outlined in the previous report, ErbB2 in this system was also constitutively active, and no effect of ASGP2 was observed on receptor activity even though the association of the two proteins in transfected cells was apparent.

The problem with both the insect and COS cell expression systems was that ErbB2 levels were too high, leading to constitutive ligand-independent activity. To circumvent this problem, we introduced ASGP2 into a human melanoma (A375) cell line that expresses very modest levels of ErbB2. ErbB2 in these cells has no detectable basal activity or tyrosine phosphorylation, but its activity may be increased markedly by treating cells with NRG1. NRG1 is a binding ligand for the ErbB3 receptor, and stimulates ErbB2 activity through ligand-stimulated receptor heterodimerization. To control for variants that might arise during the procedure of generating and selecting for stable transfectants, we introduced ASGP2 under the control of the tetracycline repression system. With this system, ASGP2 expression in stable transfectants is turned off in the presence of 2 µg/ml tetracycline. Blotting with anti-ASGP2 antibodies revealed very little ASGP2 expression under these condtions. However, when tetracycline was withdrawn, a high level of ASGP2 expression was observed both in immunoblotting and in immunofluorescent staining experiments (see Appendix). Hence, by treating cells with and without the antibiotic we could very strictly control the expression of the ASGP2 protein in a cell line where ErbB2 activation could be assessed.

To determine the effect of ASGP2 on ErbB2 kinase activity, ASGP2-transfected A375 cells were serum starved for 48 hours in the presence and absence of tetracycline. At the end of this period, cells were treated for 3 minutes without and with NRG1, cells were lysed and cleared lysates immunoprecipitated with antibodies directed to ErbB2 or ErbB3 (Appendix Figure 4A). Lysates were blotted with anti-phosphotyrosine, and then re-probed with anti-ASGP2, anti-ErbB2 or anti-ErbB3, as appropriate. We obseved that turning on the expression of ASGP2 in these cells modestly increased the extent of ErbB2 and ErbB3 tyrosine phosphorylation in the absence of added NRG1. This observation is consistent with the effect of ASGP2 on ErbB2 tyrosine kinase activity *in vitro*, and suggests that ASGP2 has some capacity to act as a cell surface autocrine ligand for ErbB receptors. However, the striking result was that ASGP2 expression dramatically potentiated the response of both ErbB2 and ErbB3 to the heterologous ligand NRG1. On the basis of these observations we now propose that rather than acting as an activating ligand for ErbB2, the function of ASGP2 is to act as a potentiating modulator of receptor function (Appendix Figure 4B, 17). Whether ASGP2 increases the intrinsic catalytic efficiency of ErbB2 or alters its substrate specificity so that it can phosphorylate residues that it could not in the absence of the modulator remains a question.

Specific Aim II: Determine whether the expression of the ASGP2/ErbB2 complex influences cellular growth or transformation.

The development of the tetracycline-inducible ASGP2 expression system was a major breakthrough because we had observed that most mammalian cultured cell lines would not support the stable constitutive expression of the protein. Also, as suggested above, this system controls for clonal variations in cell lines recovered after transfection and selection. Hence, the ASGP2-transfected A375 cells offered a powerful system for examining the biological outcome of the ASGP2/ErbB2 interaction.

To determine the effect of ASGP2 on cellular growth, we performed growth assays by measuring the incorporation of [^{3}H]thymidine into the DNA of ASGP2-transfected A375 cells in the presence and absence of tetracycline, and the presence of different concentrations of the NRG1 growth factor. In the experiment shown in Figure 1 (pg. 9), cells were serum starved for 48 hours in the presence and absence of tetracycline to simultaneous bring the cells to quiescence and to regulate ASGP2 expression. We observed that the mitogenic response of these cells to NRG1 in the presence of tetracycline is minimal (compare gray bars for None and 10 nM in the figure) relative to the serum positive control (compare gray bars for None and 10% CS in the figure). Under these conditions, where ASGP2 expression is turned off, the cells are essentially incapable of responding to the NRG1 ligand. This is probably a reflection of the very low levels of ErbB2 in these cells. However, when tetracycline was removed the cells showed a modest but reproducible increase in their basal growth rate relative to the presence of tetracycline (absence of ASGP2). This is consistent with the slight increase in constitutive ErbB receptor tyrosine phosphorylation observed when ASGP2 expression is turned on (Appendix Figure 4A). Again, the most dramatic effect was observed when NRG1 was added to the cells expressing ASGP2. While in the absence of ASGP2 A375 cells are essentially incapable of responding mitogenically to NRG1, ASGP2 expression confers NRG1 sensitivity to the cells (compare black bars for None and 10 nM). This is consistent with the observation that ASGP2 dramatically enhances the tyrosine phosphorylation of the ErbB receptors (Appendix Figure 4A), and strongly supports the contention that ASGP2 acts as a potentiating modulator for the ErbB2 receptor.

To determine whether ASGP2 might influence the localization of ErbB2, we examined the localization of the two proteins in the ASGP2-transfected A375 cells by immunofluorescence, comparing the localization of the proteins in cells treated with and without tetracycline. In the presence of tetracycline we observed essentially no staining with anti-ASGP2 antibodies, and very weak staining with anti-ErbB2 antibodies. All of the detectable ErbB2 receptor appeared to be at the cell surface. When tetracycline was withdrawn for 48 hours to induce ASGP2 expression, we observed a very strong staining of ~60% of the cells with anti-ASGP2 antibodies. Interestingly, a significant population of the cells did not express ASGP2 when tetracycline was withdrawn; the reason for this is unknown. ASGP2 also appeared to localized at the periphery of cells, suggesting a cell surface localization. Likewise, ErbB2 in the population of cells that show ASGP2 expression co-localized with ErbB2 at the cell surface, suggesting that ErbB2 did not significantly alter receptor localization. We also stained cells with an antibody that recognizes tyrsosine phosphorylated ErbB2, or the activated form of the receptor. Interestingly, the population of cells that stained positively for AS GP2 expression also stained positvely for activated ErbB2 at the cell surface. Surrounding cells that showed no ASGP2 expression did not stain positively with anti-activated ErbB2. These observations are consistent with the increased effect of ASGP2 on the basal tyrosine phosphorylation of ErbB2.

Conclusions

Our previous studies indicate that ASGP2, a cell surface protein constitutively associated with ErbB2 in an autonomously proliferating metastatic rat mammary tumor, interacts directly with ErbB2. This observation, together with the observation that ASGP2 possesses domains highly homologous to activating ligands for ErbB receptors, led us to propose that ASGP2 serves as an activating ligand for the ErbB2 receptor and should stimulate the growth of cells in which the two proteins are expressed. The goal of the studies reported here was to test this hypothesis. Using an inducible expression system, we found that ASGP2 modestly increases the tyrosine phosphorylation of ErbB2 in the absence of exogenous ligand, but strongly potentiates ErbB2 by the heterologous ligand neuregulin-1 (NRG1). Likewise, ASGP2 potentiates the growth response of cells to added NRG1 ligand. These observations suggest that rather than acting as an activating ligand, ASGP2 serves as a potentiating modulator of ErbB2 activity. Results from the laboratory of my collaborator, Dr. Kermit Carraway at the University of Miami School of Medicine, indicate that ASGP2 is expressed in approximately 70% of highly aggressive breast tumors from patient effusions, but in only 15% of ErbB2-positive solid tumors, suggsting a correlation between ASGP2 expression and breast tumor progression. Hence, ASGP2 aberrantly expressed by mammary tumor cells may synergize with endogenous or autocrine EGF-like growth factors in or surrounding the tumor to stimulate ErbB2 activity. The aberrantly activated ErbB2 may then contribute to the anomolous growth of tumor cells or may participate in other processes that contribute to breast tumor progression.

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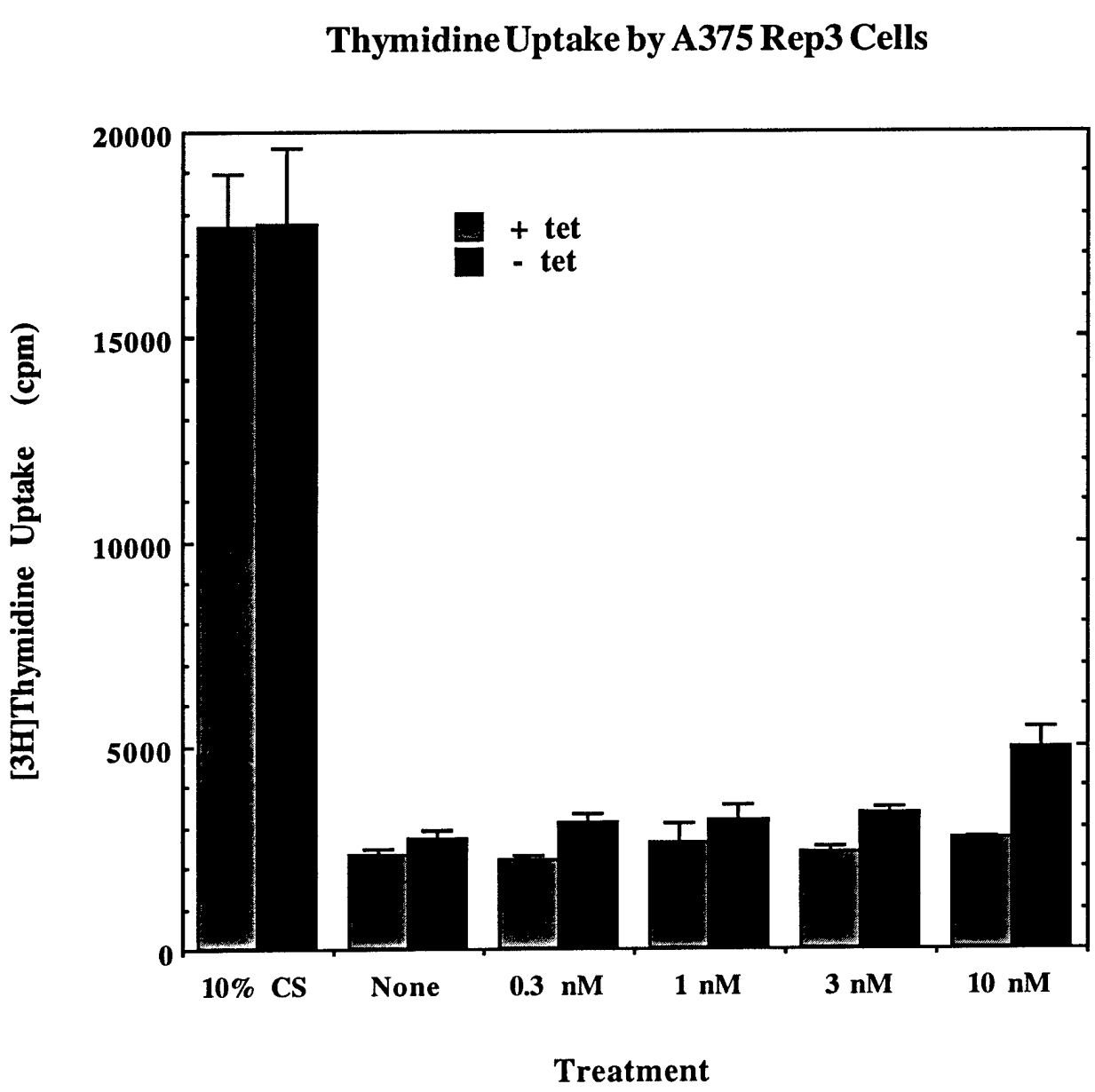
Thymidine Uptake by A375 Rep3 Cells
20000
15000
10000
5000
0
[3H]Thymidine Uptake (cpm)
+ tet
- tet
10% CS
None
0.3 nM
1 nM
3 nM
10 nM
Treatment

Figure 1

APPENDIX

An Intramembrane Modulator of the ErbB2 Receptor Tyrosine Kinase that Potentiates Neuregulin Signaling

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Abstract

The ErbB2 receptor tyrosine kinase plays a critical role in a variety of developmental processes, and its aberrant activation may contribute to the progression of some breast and ovarian tumors. ASGP2, a transmembrane glycoprotein found on the surface of the highly metastatic ascites 13762 rat mammary adenocarcinoma cell line, is constitutively associated with ErbB2 in these cells and in mammary tissue from pregnant rats. Expression studies indicate that ASGP2 interacts directly and specifically with ErbB2 through one of its EGF-like domains, and that the co-expression of the two proteins in the same cell dramatically facilitates their direct stable interaction. Ectopic expression of ASGP2 in human melanoma tumor cells potentiates the response of endogenous ErbB2 to the Neuregulin-1 growth factor. These observations point to a novel intramembrane mechanism for the modulation of receptor tyrosine kinase activity.

ErbB2 (also known as Neu) is a 185 kD cell surface transmembrane receptor tyrosine kinase that mediates the growth or differentiation of a variety of cultured cells and contributes to the proper development of cardiac and neural tissues during gestation (1). Its overexpression in numerous human tumors, including breast and ovarian tumors, correlates with earlier patient relapse and poor prognosis (2). The observation that ErbB2 overexpression stimulates its protein tyrosine kinase activity (3), together with the observation that activated alleles of the *erbB2* gene induce metastatic tumors when expressed in murine mammary epithelium (4), suggest that the activation of ErbB2 kinase activity may play an important role in tumorigenesis or tumor progression.

The protein tyrosine kinase activity of ErbB2 may be activated by several soluble, diffusible ligands that possess epidermal growth factor (EGF)-like domains. For example, EGF, transforming growth factor-α (TGF-α) and amphiregulin (AR) are all capable of stimulating ErbB2 activity by binding to the related EGF receptor and promoting its heterodimerization with ErbB2 (5). Likewise, the neuregulins (NRGs) bind to the ErbB3 and ErbB4 receptors and stimulate ErbB2 activity through receptor heterodimerization mechanisms (6). However, no molecularly characterized diffusible ligand has been demonstrated to act on ErbB2 directly, and it has been suggested that the primary function of this protein is to augment signaling through the ErbB receptor network by acting as an auxiliary co-receptor (7). In this context factors that influence the activity or availablity of ErbB2 could have a significant impact on the strength or specificity of signaling and ultimately the cellular response. Strong candidates for such factors are cell surface proteins that possess EGF-like domains.

The autonomously proliferating and highly metastatic rat ascites 13762 mammary adenocarcinoma cell line expresses a large sialomucin complex in abundance at its cell surface. This complex consists of two non-covalently associated proteins, ASGP1 and ASGP2, which arise from the proteolytic processing (8) of the product of a single gene (9). ASGP1, a ~600 kD heavily O-glycosylated sialomucin, is an anti-adhesive factor as well as a contributor to the ability of these

cells to evade immune recognition (10, 11). The 120 kD transmembrane subunit ASGP2 tethers the complex to the cell surface. The sequence of ASGP2 includes two EGF-like domains, one of which conserves all of the consensus residues of the active EGF-like growth factors (9). 13762 ascites cells also express at their surfaces modest levels of ErbB2. The receptor and several of its associated intracellular signaling proteins are constitutively tyrosine-phosphorylated in the ascites cells, suggesting that ErbB2 is constitutively activated (12).

To test the whether ASGP2 might act as a modulator of ErbB2 function, the association of the two proteins in 13762 cells was first examined by co-immunoprecipitation. Detergent solubilized plasma membranes (13) were immunoprecipitated with either anti-ASGP2 or anti-ErbB2, and precipitates were immunoblotted with antibodies to the other (14). ASGP2 was observed in anti-ErbB2 immunoprecipitates, and ErbB2 was observed in anti-ASGP2 precipitates (Fig. 1A). Since the immunoprecipitations were performed using fractions isolated from microvillar plasma membranes (13), these findings indicate that ASGP2 and ErbB2 are present in a complex on the surface of 13762 cells. We have previously observed that during pregnancy the expression of ASGP2 in the mammary epithelium of rats increases dramatically, and a fraction of the expressed sialomucin complex is secreted into milk (15). As with the 13762 cells, ASGP2 and ErbB2 could also be co-immunoprecipitated from lysates of homogenized mammary tissue from animals 17 days pregnant (15; Fig. 1B), suggesting that their association is a normal physiological event and not a result of the aberrant overexpression of ASGP2 in the tumor cells.

Although the observations above indicate that ASGP2 and ErbB2 are constitutively associated at the surface of 13762 cells, the participation of another receptor or other proteins in the interaction could not be ruled out. To test the specificity of the ASGP2/ErbB2 interaction a baculovirus/insect cell expression system was developed. Insect cells were employed because they do not express endogenous ErbB receptors, eliminating confusion arising from potential receptor heterodimerization events. In the first series of experiments Sf9 insect cells were infected with

baculovirus encoding ASGP2 alone, or co-infected with ASGP2 and each of the known ErbB receptors (16). The co-immunoprecipitation assay was used to assess association. It was observed that ASGP2 could be co-immunoprecipitated with ErbB2 from cells expressing both proteins, but could not be co-precipitated with the EGF receptor, ErbB3 or ErbB4 proteins (Fig. 2A). Likewise, ASGP2 could be co-immunoprecipitated with ErbB2 when the two proteins were transiently co-expressed in COS cells, but could not be co-precipitated with the endogenous COS cell EGF receptor (data not shown). These observations indicate that the stable association of ASGP2 with ErbB receptors is selective for ErbB2 and does not require another ErbB receptor. Deletion analysis indicated that, as expected, the extracellular domain of ErbB2 is necessary for its interaction with membrane-bound ASGP2. When co-expressed in SF9 cells, ASGP2 could be co-immunoprecipitated with either full-length ErbB2 or the extracellular domain of the receptor, but could not be co-precipitated with the intracellular domain or a transmembrane form lacking most of the extracellular domain (Fig. 2B).

The extracellular domains of the ErbB2 and ASGP2 proteins could also be secreted as a complex when co-expressed in the same cell. In the experiment shown in Fig. 3A, High Five insect cells (cells specifically adapted for the expression of secreted proteins) were infected with baculovirus encoding the extracellular domain of ASGP2 (ASGP2 ECD) or the extracellular domain of ErbB2 (Neu ECD), or were co-infected with both viruses (17). The co-immunoprecipitation assay was then carried out with the cleared conditioned media from infected cells using anti-ErbB2 antibodies. ASGP2 ECD was detected in immunoprecipitates from cells expressing both proteins, indicating that the cells secrete ASGP2 ECD and Neu ECD as a complex. Similar immunoprecipitates from metabolically labeled cells showed no other detectable radiolabeled bands (18), suggesting that the ASGP2/ErbB2 association occurs through a direct protein-protein interaction. Moreover, resolution of the radiolabeled immunoprecipitated proteins by non-reducing SDS-PAGE demonstrated that the association of ASGP2 and ErbB2 is non-covalent in the secreted complex

from the High Five cells. Finally, sedimentation analysis of the secreted complex suggested that ASGP2 is capable of associating in a 1:1 complex with monomeric Neu ECD (18).

To determine the domain within ASGP2 that mediates its interaction with ErbB2, deletion mutagenesis from the carboxy terminus of the ligand was performed. ASGP2 deletion mutants were co-expressed with Neu ECD in High Five cells, and the co-immunoprecipitation assay was employed to determine the extent of interaction between the expressed secreted proteins (Fig. 3B). ASGP2 forms containing EGF1, the EGF-like domain that possesses the consensus residues found in active growth factors (9), could be co-immunoprecipitated with Neu ECD. However, when the EGF1 domain was deleted, the ability of ASGP2 to associate with ErbB2 was almost completely abolished. These results indicate that the EGF1 domain of ASGP2 is necessary for its stable interaction with the ErbB2 receptor.

Fig. 3A demonstrates that the co-expression of the ASGP2 and ErbB2 proteins is necessary for their interaction. When the conditioned media from insect cells independently expressing ASGP2 ECD and Neu ECD were mixed, no co-immunoprecipitation of ASGP2 and ErbB2 was observed. This is consistent with our observations that ASGP2 ECD expressed in either insect cells or COS monkey cells will neither bind to nor activate ErbB2 when added exogenously to cultured mammalian cells that express this receptor (19). The reason for the requirement of co-expression for ASGP2/ErbB2 complex formation is presently unclear. The simplest explanation is that high concentrations of ASGP2 and ErbB2 are necessary for complex formation, a condition which is met in membranes and in cellular compartments, but not by the addition of soluble ligand to cells. Once formed, the complex is very stable and resistant to dissociation during the immunoprecipitation procedures.

The results above indicate that ASGP2 binds directly to the ErbB2 receptor when the two proteins are co-expressed in the same cell. To examine the functional outcome of the interaction,

sialomucin complex was expressed in an inducible manner in A375 human melanoma cells (20). These cells express modest levels of ErbB2 and ErbB3, and respond biochemically to the Neuregulin-1 (NRG1) growth factor ligand. When ASGP2 expression was turned on with the removal of tetracycline from the growth media, it was observed that the basal and NRG1-stimulated tyrosine phosphorylation of the ErbB2 and ErbB3 receptors was increased substantially (Fig. 4A). However, blotting with anti-ErbB2 and anti-ErbB3 antibodies indicated that this increase could not be accounted for by an increase in overall receptor expression. These observations suggest that a functional outcome of the interaction of ASGP2 with ErbB2 is to potentiate its response to EGF-like ligands.

We conclude that when co-expressed in the same cell, ASGP2 interacts directly with ErbB2 extracellular domain through its EGF1 domain and potentiates signaling through the ErbB receptor network. Interestingly, ASGP2 affects the extent of NRG1-stimulated receptor tyrosine phosphorylation rather than the dose-response curve of activation (21), implying that its role is not in the facilitation of ErbB2/ErbB3 heterodimeric complexes. Our results instead suggest that ASGP2 increases either the number of ErbB2 molecules available for activation or the extent of activation of each ErbB2 receptor (Fig. 4B).

In ascites 13762 cells, ASGP2 association could contribute to the constitutive tyrosine kinase activity of ErbB2 (12), which in turn may contribute to the autonomous growth properties or the malignancy of these cells. A question that naturally arises is whether the sialomucin complex is involved in human breast cancer. In preliminary studies (22), sialomucin complex was found in approximately 70% of highly aggressive breast tumors from patient effusions, but in only 15% of ErbB2-positive solid tumors, perhaps suggesting a role in tumor progression. Further studies are in progress to substantiate these findings and to determine whether sialomucin complex may have significance in prognosis or as a target for therapy.

References and Notes

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13. Ascites 13762 adenocarcinoma cells (MAT-C1 subline) were grown intraperitoneally in Fischer 344 rats, and microvillar membranes were prepared under microfilament-depolymerizing conditions as described previously [K. L. Carraway, J. W. Huggins, R. F.

Cerra, D. R. Yeltman, C. A. C. Carraway, *Nature* **285**, 508 (1980); K. L. Carraway, R. F. Cerra, G. Jung, C. A. C. Carraway, *J. Cell Biol.* **94**, 624 (1982)]. Microvilli (5.0 ml, ~15 mg/ml protein) were incubated for 30 min at 4°C in 5 mM glycine, 2 mM EDTA, 2 mM dithiothreitol, pH 9.5, and then homogenized in a Dounce B homogenizer. After centrifugation at 10,000xg the membranes were harvested and washed using centrifugation at 150,000xg for 1.5 hr. Microvillar membranes were solubilized in S buffer (0.2% Triton X-100, 150 mM KCl, 2 mM $MgCl_2$, 0.2 mM ATP, 0.2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 5 mM Tris, pH 7.6) and centrifuged on 7-25% sucrose gradients in S buffer for 15 hr at 80,000xg and 4°C. Gradients were fractionated and prepared for SDS-PAGE and immunoblotting. Selected fractions were combined and analyzed by immunoprecipitation (14).

14. Immunoprecipitations of rat ErbB2 or its deleted variants were carried out with 1 μg of either Ab-3 or Ab-4 anti-ErbB2/Neu monoclonal antibodies (Oncogene Science) using 2 μg rabbit anti-mouse IgG secondary antibody (Zymed) to bind to Protein A-Sepharose. Immunoprecipitations of ASGP2 were carried out with 3 μl polyclonal anti-ASGP2 (8). Immunoblotting using anti-ErbB2 Ab-3, polyclonal anti-ASGP2 (8) or monoclonal anti-ASGP2 (15) was performed using either alkaline phosphatase or enhanced chemiluminescence for detection.

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16. Sf9 insect cell growth, infections and transfections were carried out as previously described [P. M. Guy, K. L. Carraway III and R. A. Cerione, *J. Biol. Chem.* **267**, 13851 (1992); K. L. Carraway III *et al.*, *J. Biol. Chem.* **269**, 14303 (1994)]. In co-expression experiments where a single protein was expressed as a control, wild type baculovirus was used as the co-infecting virus. Sf9 cell lysis was performed using an NP-40 lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 1% NP-40, 1mM EDTA, 1 mM orthovanadate, 100 μM leupeptin, 20 KIU/ml aprotinin, 1 mM PMSF, 1 mM benzamidine). Lysates were cleared by centrifugation at 12,000xg for 15 minutes prior to immunoprecipitation.

17. Infections of High Five cells were performed with $2x10^6$ cells/well of 12-well tissue culture dishes and incubation at 27°C. Cells were incubated for 24 hours with baculovirus containing DNA encoding Neu ECD and the ASGP2 deletion mutants at a multiplicity of infection between 5 and 10 for each virus. The media were replaced with Excell 405 serum free media (JRH Biological). The conditioned media were collected after an additional 32 hours and clarified by centrifugation at 12,000xg. An equal volume of 2X RIPA buffer was added prior to immunoprecipitation.
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19. K. L. Carraway III, E. A. Rossi, K. Masanobu and K. L. Carraway, unpublished observations.
20. Construction of A375 human melanoma cell lines expressing sialomucin complex under tetracycline regulation has been described previously (10). Cells were grown to 80% confluence in the presence of tetracycline, and concomitantly serum starved in 0.1% calf serum and treated with and without 1 μg/ml tetracycline for 48 hours. Cells were then treated without and with purified NRG1β1 EGF-like domain [K. L. Carraway, III *et al.*, *Nature* **387**, 512 (1997)] for two minutes, lysed in NP-40 lysis buffer, and cleared lysates were immunoprecipitated with antibodies to ErbB2 (Ab-3) or to ErbB3 [3184; K. L. Carraway, III, S. P. Soltoff, A. J. Diamonti, L. C. Cantley, *J. Biol. Chem.* **270**, 7111 (1995)]. Precipitates were analyzed first by blotting with anti-phosphotyrosine, and then by cutting apart the filter and reprobing with anti-ASGP2, anti-ErbB2 and anti-ErbB3.
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23. We thank C. Ivanof, A.J. Diamonti and E. Hanson for technical assistance. Supported by grants from the Massachusetts Department of Public Health Breast Cancer Program and Department of Defense Breast Cancer Research Program grant DAMD17-96-1-6082

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Figure Legends

Fig. 1. *In vivo* association of ASGP2 and ErbB2 demonstrated by co-immunoprecipitation. **(A)** Cleared lysates containing plasma membrane proteins from ascites 13762 rat mammary adenocarcinoma cells were immunoprecipitated and immunoblotted with anti-ErbB2 or anti-ASGP2 as indicated. **(B)** Cleared detergent homogenates from the mammary tissue of lactating rats 17 days pregnant were immunoprecipitated with anti-ErbB2 and blotted with the indicated antibodies. In both experiments lanes marked 'none' represent lysates used as positive controls for blotting.

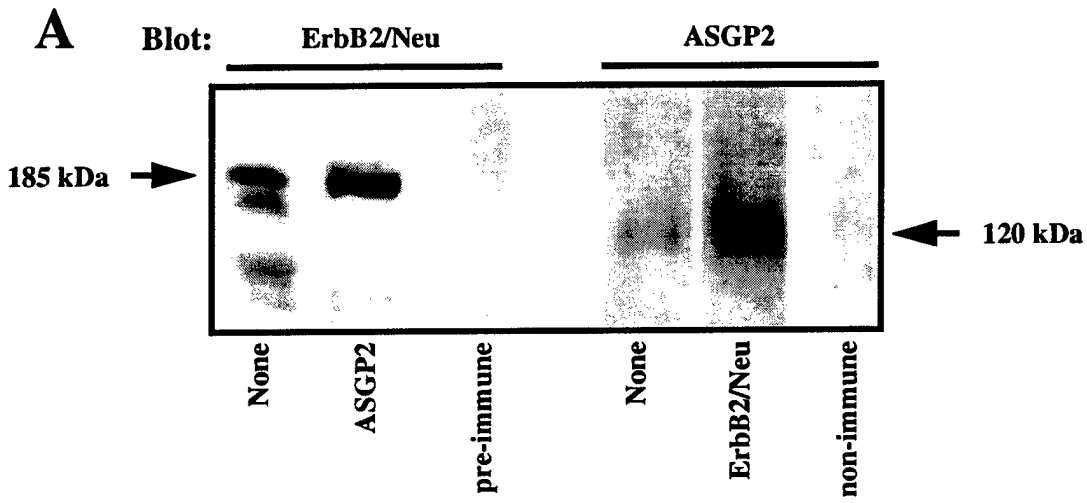
Fig. 2. Specific association of ASGP2 with ErbB2 extracellular domain. **(A)** Sf9 insect cells were co-infected with viruses encoding ASGP2 and each of the known ErbB receptors, as indicated. Cell lysates were immunoprecipitated with appropriate anti-receptor antibodies and precipitates were blotted with anti-ASGP2. The left two lanes represent control immunoprecipitations from cells expressing ASGP2 alone with anti-EGFR and anti-ErbB2, respectively. **(B)** Cells were co-infected with baculovirus encoding ASGP2 and the illustrated ErbB2 constructs (upper panel), and the co-immunoprecipitation assay was used to assess association (lower panel). Lysates immunoprecipitated with appropriate anti-ErbB2 antibodies were blotted with anti-ASGP2. The right two lanes represent control immunopreciptations with the two anti-ErbB2 antibodies employed in the experiment. In both experiments, Ponceau S staining of the filters indicated that similar levels of receptors were present in each of the immunoprecipitates (not shown).

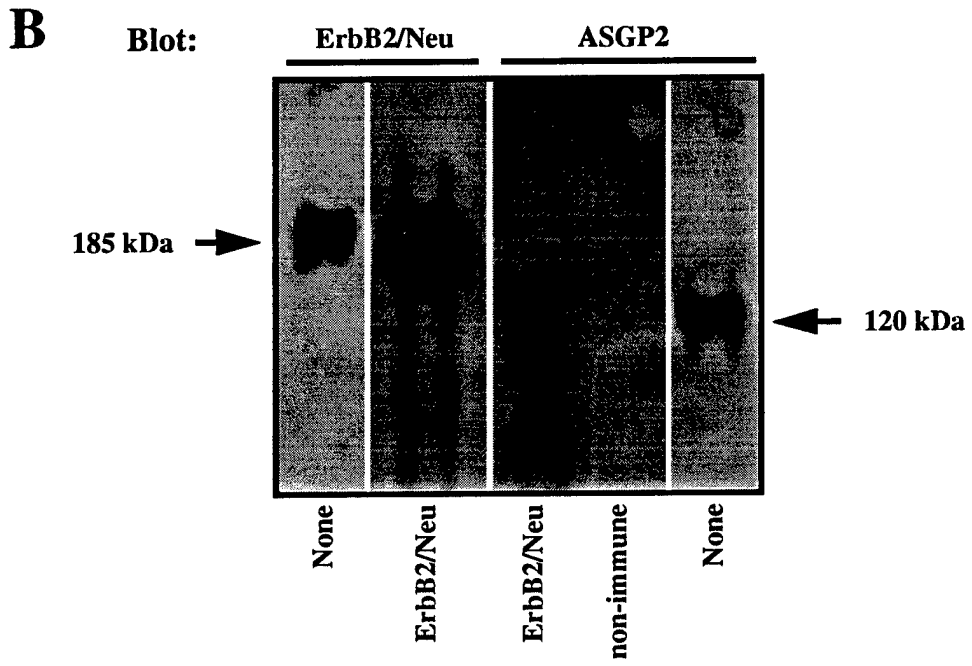
Fig. 3. Association of ASGP2 and ErbB2 extracellular domains. **(A)** High Five insect cells were infected with virus encoding the extracellular domain of ErbB2 (Neu ECD, lanes A), virus encoding the extracellular domain of ASGP2 (ASGP2 ECD, lanes B) or co-infected with the two viruses (lanes C). Equal volumes of the conditioned media for the Neu ECD and ASGP2 ECD

individual infections were also mixed (lanes D). Conditioned media and immunoprecipitates using anti-ErbB2 or anti-ASGP2 were blotted with anti-ASGP2 after SDS-PAGE. **(B)** High Five cells were co-infected with baculovirus encoding Neu ECD and the illustrated ASGP2 constructs (upper panel), and association was measured by co-immunoprecipitation (lower panel, left) from the conditioned media (lower panel, right) and immunoblotting with anti-ASGP2.

Fig. 4. Effect of ASGP2 expression on ErbB2 activity. **(A)** A375 human melanoma cell transfectants were treated with and without tetracycline to induce sialomucin complex expression, and then treated without and with recombinant NRG1β1, as indicated. Lysates, anti-ErbB2 and anti-ErbB3 immunoprecipitates were first blotted with anti-phosphotyrosine (upper panel). The filter was then cut apart and lysate lanes (lower left panel), anti-ErbB2 lanes (lower middle panel) and anti-ErbB3 lanes (lower right panel) were re-probed with anti-ASGP2, anti-ErbB2 and anti-ErbB3, respectively. **(B)** Model for ASGP2 potentiation of NRG1 signaling. In cells lacking ASGP2 expression (left), a trimeric complex of ErbB2, ErbB3 and NRG mediate normal signaling. In ASGP2-expressing cells (right), a tetrameric complex of ASGP2/ErbB2, ErbB3 and NRG becomes hyperphosphorylated sending an augmented or different signal to the cell.

Carraway et al., Fig. 1





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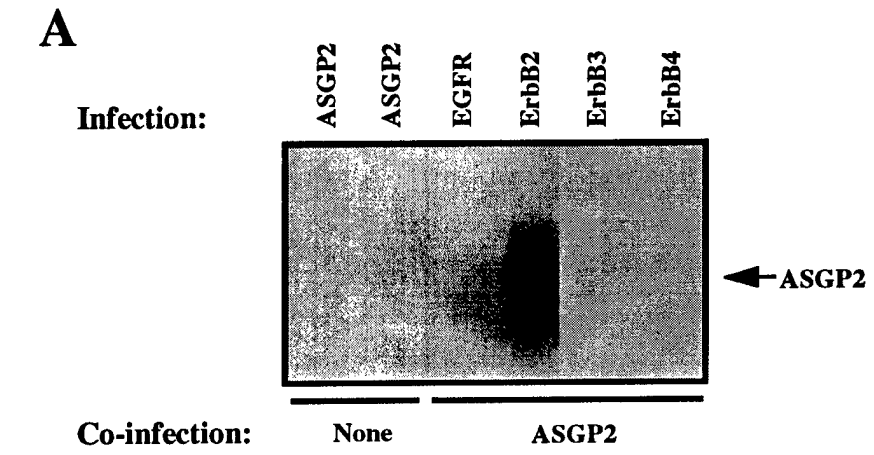
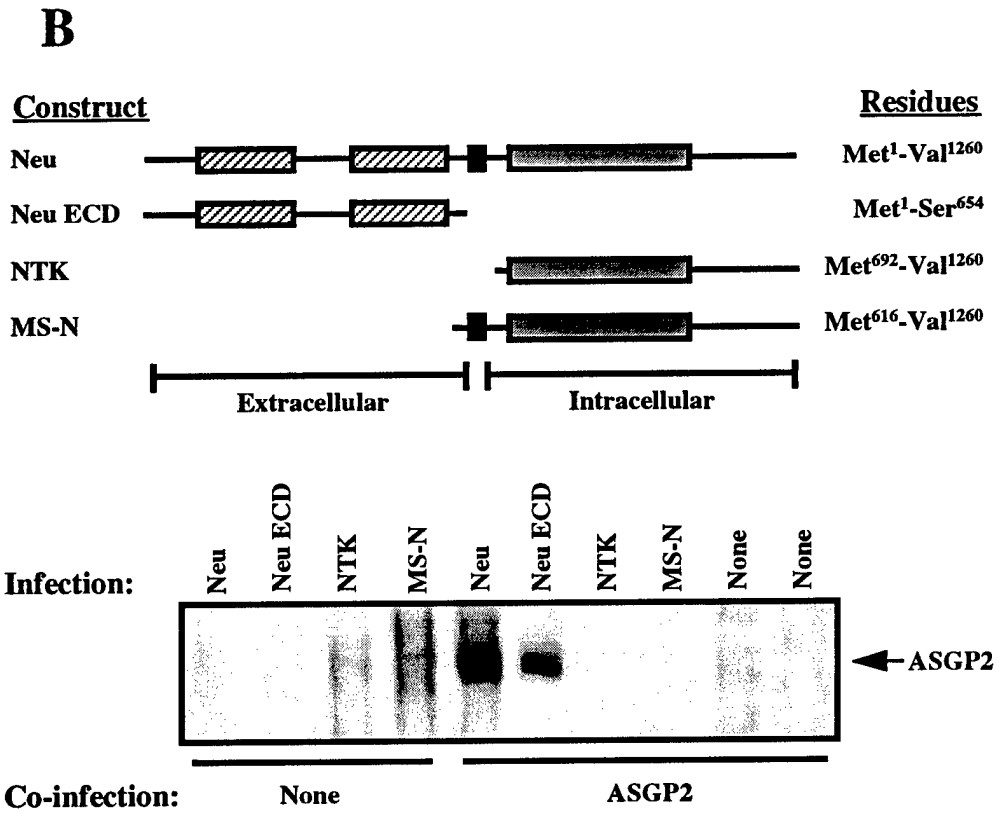
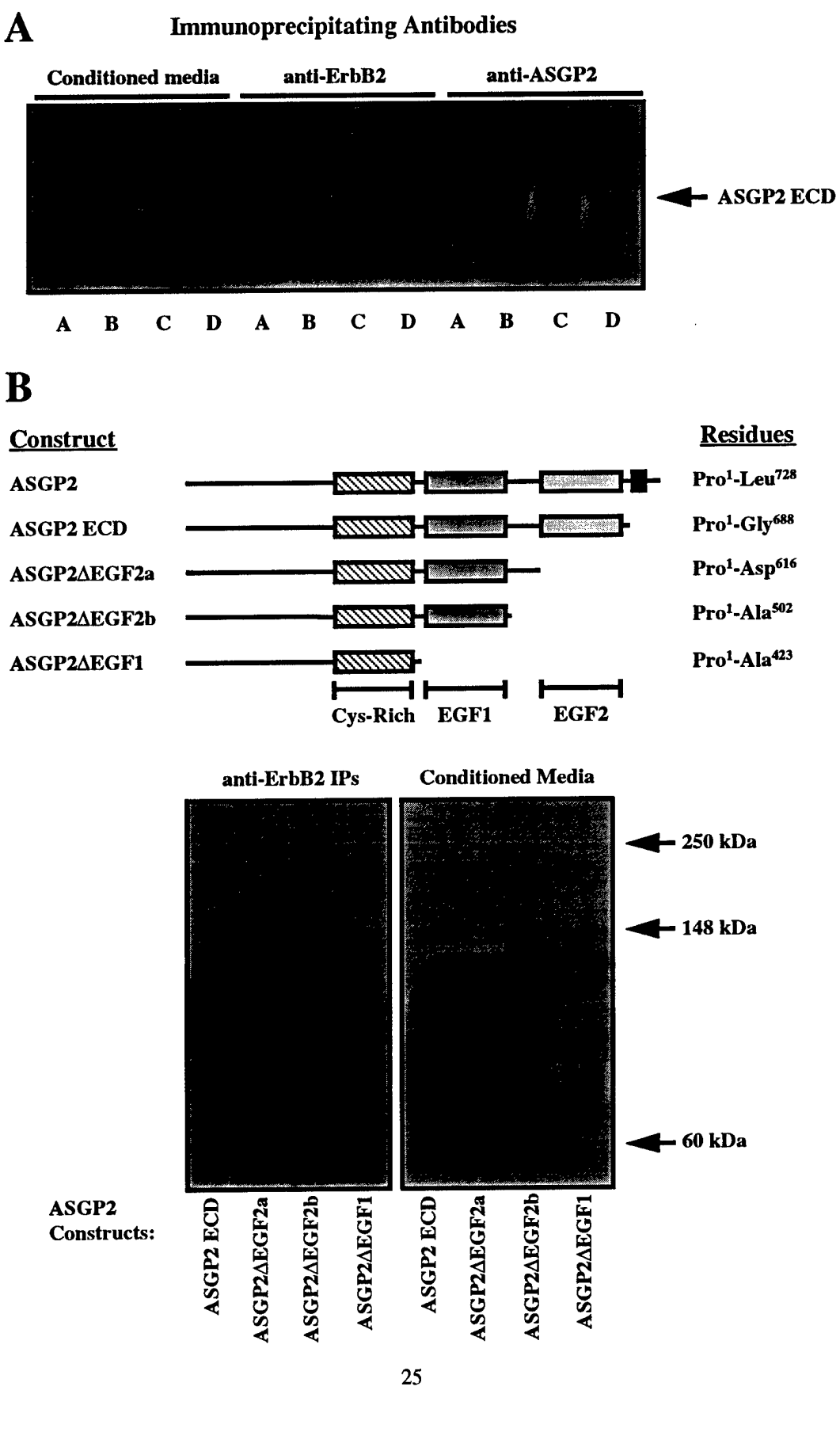




Carraway et al., Fig. 3



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